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Catching Lipid Droplet Contacts by Proteomics

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Abstract

Lipid droplets (LDs), important organelles for energy storage and involved in the development of metabolic disorders, are extremely dynamic and interact with many other cellular compartments to orchestrate lipid metabolism. Little is known about how these organelle contacts are changed according to cellular needs and functions under different metabolic and pathological conditions and which proteins regulate this. Here, we summarize recent exciting discoveries about the reorganization of organelle contacts in steatotic liver, including the identification of novel LD contact site proteins in cell lines and in animals. We also discuss state of the art proteomics workflows that enable the characterization of LD-organelle contacts and tethering proteins and give an outlook how this can inform obesity research.

Keywords

lipid droplet; proteomics; phosphoproteome; contact site; nonalcoholic fatty liver disease; steatosis

Lipid Droplets—An Extremely Dynamic and Connected Organelle

Cellular organelles are highly dynamic structures that adapt their protein and lipid composition, biochemical activities, cellular abundance, and distribution but also their interactions with each other to environmental challenges. Nutrient availability is one of the environmental factors with the highest fluctuations. Normally, cells cope well with changing nutrient levels that organisms are exposed to on a daily basis such as fasting and feeding cycles. However, chronic caloric overexposure represents a severe form of environmental

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stress. Excessive energy uptake leads to increased lipid synthesis and the accumulation of free fatty acids and toxic lipid species such as diacylglycerols or ceramides. Their sequestration in the form of neutral lipids in lipid droplets (LDs) provides a mechanism to prevent lipotoxicity (Chaurasia and Summers, 2015; Engin, 2017). LDs are essential for normal energy metabolism, mediate many metabolic processes, and provide building blocks for membranes. However, LD accumulation, especially in tissues and cell types not specialized for lipid storage such as macrophages or the liver, is a hallmark of the development of many diseases such as atherosclerosis or hepatic steatosis (Krahmer et al., 2013a). Therefore, elucidating the regulation and dynamics of LD composition and interactions is indispensable to understand mechanisms underlying disease development.

LDs are extremely dynamic organelles that fuse, undergo fission, and adapt their size several orders of magnitude according to metabolic demands. LDs are unusual organelles inasmuch as they consist of a lipid core, including triglycerides or sterol esters, which is separated by a phospholipid (PL) monolayer, rather than a bilayer, from the aqueous cytosol (Olzmann and Carvalho, 2018). The LD proteome is highly dynamic and diverges between tissues, cell types, and metabolic conditions (Yu et al., 2015; Bersuker et al., 2018). Proteins can bind to the LD surface monolayer via hydrophobic elements, such as amphipathic helices, hydrophobic domains, or lipid modifications but do not require a specific targeting sequence (Kory et al., 2016). Many proteins have structural capacities to attach to LDs; however, not all of these proteins localize to LDs or they do so only under certain conditions. Only few LD marker proteins localize exclusively to LDs and many of them have multiple cellular localizations with only a certain pool targeted to LDs (Kory et al., 2015). How the LD targeting is regulated and specificity of LD binding is achieved is still poorly understood and the regulation of the LD proteome is likewise largely enigmatic.

One crucial question is how LD proteins distinguish between a lipid bilayer and the LD monolayer. Computer simulations revealed that the PL monolayer differs from bilayers by the presence of large and persistent lipid packing defects, which are discontinuities in the PL layer induced by the underlying neutral lipid layer. At these sites, neutral lipids are exposed on the surface inducing energetically unfavorable interactions between the hydrophobic lipid patches and the hydrophilic cytosol (Bigay and Antonny, 2012). Many cytosolic proteins with amphipathic helices containing large hydrophobic residues can detect and bind these sites to a different extent, depending on the length and size of hydrophobic residues within the amphipathic helices. However, the fact that only a minor pool of these amphipathic helices containing proteins binds to LDs under normal cellular conditions, or some proteins only target a specific pool of LDs (Thul et al., 2017), suggests that there are additional mechanisms that selectively prevent proteins from binding LDs, such as protein–protein interactions or binding competition to other cellular membranes. Another possible mechanism controlling the LD proteome is macromolecular crowding at the LD surface. Normally, the number of LDs and hence binding surfaces on the PL monolayer are limited, so that proteins with weaker affinities are crowded off the LD surfaces and only proteins with stronger affinities can bind (Prevost et al., 2018).

LD associations with almost all other cellular compartments including the endoplasmic reticulum (ER), Golgi, mitochondria, lysosomes, and peroxisomes have been observed by

different techniques such as multispectral time-lapse microscopy, super-resolution microscopy, or electron microscopy (Pu et al., 2011; Valm et al., 2017; Gemmink et al., 2018). these contacts might serve various cellular functions such as protein transport, substrate flux, and orchestration of lipid metabolism (Schuldiner and Bohnert, 2017). To promote lipid storage, membrane bridges with the ER are formed, allowing transfer of lipid synthesis enzymes onto LDs (Wilfling et al., 2013). In addition, contacts to mitochondria or peroxisomes were suggested to coordinate lipid oxidation (Boutant et al., 2017), and accumulation of LDs around mitochondria was reported to protect these from toxic lipid species (Nguyen et al., 2017). In addition, close proximity of mitochondria and LDs in brown adipocytes favor lipid synthesis, storage, and LD expansion by providing ATP for fatty acid activation and resulting in triglyceride synthesis. This is supported by the fact that in brown adipocytes, LD-associated mitochondria exhibit reduced β -oxidation and increased ATP synthesis (Benador et al., 2018).

As for most other organelle interactions, the presence of tethering proteins or complexes between LDs and the other organelles has been postulated (Eisenberg-Bord et al., 2016). these tethers are required to keep opposing membranes in close proximity and might have additional functions in lipid and metabolite transfer, protein shuttling, or signal transduction. However, the field is just beginning to reveal the identity of those LD contact site proteins and their roles in regulating organelle interactions according to metabolic and physiological needs. The functional analysis of these tethering complexes is complicated by the fact that some of them might have overlapping and redundant functions and that they may only fulfill their tethering function under certain metabolic conditions or in specific tissues. The metabolic context is hard to study in cell lines because complex metabolic changes such as overnutrition or starvation are difficult to simulate. In vivo, cells are embedded in their complex organ environment, exposed to organ crosstalk, metabolic hormones, and complex diets. This is difficult to mimic with commonly used approaches such as loading cells with fatty acids or inducing serum starvation.

In the last years, first bona fide LD tethering factors have been identified. As an example, the seipin complex forming an oligomeric ring at LD-ER junctions tethers newly forming LDs to the ER (Sui et al., 2018; Yan et al., 2018). In cells with seipin mutations, ER-LD interactions are altered, suggesting that seipin is involved in the formation of membrane bridges that normally connect the two organelles (Salo et al., 2016).

Recently, an LD binding domain has been mapped on VPS13A/C. VPS13 proteins are involved in various organelle contacts in yeast and mammals (John Peter et al., 2017; Bean et al., 2018). The VPS13A isoform primarily localizes to ER-mitochondria contacts, whereas VPS13C localizes to contacts between ER and endosomes (Kumar et al., 2018). ER association happens via the interaction of its N-terminal domain with VAMP-associated protein-proteins, whereas its C-terminal regions mediate endosome/LD and mitochondria/LD binding. Interestingly, the highly conserved N-terminal portion of VPS13 is tubular, with a large hydrophobic cavity that can solubilize and transport PLs between membranes. VPS13 proteins might play a role for PL transfer at ER-LD contacts and may therefore represent the long thought pathway that supplies growing LDs with PLs (Krahmer et al., 2011).

Identification of the LD Proteome, Interactions, and Contact Site Proteins by Proteomic Profiling

In the last decades, the LD proteome of cell lines and tissues has been extensively studied (Cermelli et al., 2006; Bouchoux et al., 2011; Schmidt et al., 2013; Baumeier et al., 2015; Khan et al., 2015; Kramer et al., 2018). Most experimental approaches have so far relied on determining proteins present in a purified LD fraction. However, as for other organelles, it has become obvious that it is impossible to purify LDs to complete homogeneity, especially as the tight membrane interactions cause cofloation of various organelles or their fragments. Therefore, and especially with the increasing sensitivity of mass spectrometry (MS), the detection of a protein in a purified LD fraction is insufficient evidence of its specific LD association and does not allow to distinguish genuine LD proteins from contaminants. In cell lines, this problem can be overcome by using proximity labeling strategies, such as APEX or BioID. Here, by expression and activation of a peroxidase-based proximity tag, proteins within a certain distance (~10 nm) can be covalently modified with an affinity handle that can subsequently be used to enrich bona fide organellar proteins (Bersuker et al., 2018).

Spatial proteomics profiling approaches can also be used to discriminate compartment-associated proteins from unspecific contaminants. These methods are based on a separation and partial enrichment of certain organelles depending on their size and density either by differential centrifugation or on a density gradient (Figure 1). Subsequent MS-based analysis of the fractions allow the generation of abundance profiles for the quantified proteins reflecting their subcellular distributions and subcellular localizations. Although organelle fractions are not pure, the distribution of proteins from a specific compartment over all fractions is highly characteristic for each organelle. The quantitative profile of organellar marker proteins indicates the profile for each of them. Protein correlation profiling (PCP) has been used successfully to determine the proteomes of many organelles including ones that could never be purified before (Andersen et al., 2003; Wiese et al., 2007; Pandya et al., 2017), but also for systematic subcellular maps, even in comparative manner (Dunkley et al., 2004; Foster et al., 2006; Itzhak et al., 2016; Geladaki et al., 2019), thereby generating comprehensive views on cellular processes under specific biological or pathological conditions.

Advances in MS-based technologies have pushed further the development and quality of proteomic profiling approaches over the last years. Label-free quantification algorithms are increasing the accuracy of protein profiles (Cox et al., 2014). Multiplexing of proteomic samples by using isobaric tags such as tandem mass tags (TMT) (Rauniyar and Yates, 2014; Washburn et al., 2002) or EASI-tag (Easily Abstractable Sulfoxide-based Isobaric tag) (Virreira Winter et al., 2018) can help to overcome problems with missing values, increase accuracy, and reduce the required MS measuring time. In addition, bioinformatic tools such as machine learning-based determination of subcellular localizations have strongly facilitated and improved the quality of organelle assignments (Itzhak et al., 2016; Crook et al., 2018). Novel high sensitivity MS-based proteomic workflows including improved sample preparation methods, more sensitive instruments, and scan modes now allow the

generation of high quality profiles for large numbers of proteins, increasing the number of mapped proteins and extending the dynamic range of localized proteins.

The development of phosphoproteomic workflows enabling high-throughput quantification of phosphopeptides from small amounts of protein input such as organelle fractions make it feasible to extend organelle maps to phosphopeptide level for the first time (Krahmer et al., 2018). Overlay of protein with phosphopeptide profiles allow the identification of phosphorylation events that occur only on a certain compartment. these organelle-specific phosphorylation events, especially these whose levels are correlated with protein relocalization events, might either induce relocalization or be caused by organelle-specific interactions with kinases or phosphatases. In the future, this approach could be expanded to other post-translational modifications (PTMs), such as ubiquitination or acetylation.

Protein profiling approaches have also turned out to be an useful tool to study the LD proteome of yeast or drosophila cells (Krahmer et al., 2013b; Currie et al., 2014) and especially in tissues where proximity labeling is not feasible (Krahmer et al., 2018). Due to their specific hydrophobic characteristics, LDs have a unique floating and fractionation behavior, resulting in a highly specific protein profile with a single sharp peak for LD proteins in the lowest density fraction. LD proteins with dual localizations or those enriched in contact sites display profiles with two defined characteristic organelle peaks. In addition, PCP allows the characterization of systemic reorganization of LD contacts and the detection of increased contacts with LDs by shifts and increased LD peaks in the average organelle profiles. As an example, the expression of a toxic Huntingtin form with an extended poly-Q stretch in yeast leads to a strong LD and mitochondrial phenotype and PCP revealed a loss of LD–mitochondria interaction (Gruber et al., 2018).

Unexpected Contacts in Nonalcoholic Fatty Liver Disease

Diet-induced nonalcoholic fatty liver disease affects 25% of the total population worldwide and is characterized by excessive hepatic LD accumulation (Rinella, 2015). LD formation is initially thought to be protective, but long-term effects of extreme LD accumulation are still not clear. LDs can cover up a large portion of the total cellular volume and whether this affects cellular processes and functions is unknown. Moreover, cellular mechanisms underlying the shift from reversible asymptomatic steatosis to nonalcoholic steatohepatitis, characterized by fibrosis and inflammation in some patients, remain to be elucidated (Rinella, 2015).

In a recent study by our group, proteomic profiling revealed a comprehensive overview of protein localization, phosphorylation, and subcellular organization during hepatic lipid accumulation (Krahmer et al., 2018). Diet-induced steatosis in high fat diet (HFD) mice leads to a progressive increase in the number of relocalized proteins over time and to increased LD-organelle contacts under severe steatosis. We reasoned that increased cofractionation of the plasma membrane, mitochondria, and the ER with LDs in the low-density fraction of the gradient in steatotic liver might derive from enhanced organelle associations. Indeed, increased LD-mitochondrial contacts were confirmed by electron and fluorescence microscopy. Concomitantly, several contact site proteins relocalized to LDs and

therefore are potential candidates to control these organelle associations. ESYT2, a protein channeling lipids between the plasma membrane and ER, partially shifted to LD localization, and VPS13A/D displayed a dual LD/mitochondrial peak. However, VPS13A might not mediate binding of LDs to mitochondria but instead simultaneous binding of LDs and mitochondria to the ER, as the LD and mitochondria binding domains of VPS13A are in close proximity of each other at the C-terminal part of the protein and likely exclude each other (Kumar et al., 2018). Increased contacts between mitochondria and ER were observed in the liver of HFD mice (Arruda et al., 2014). This raises the question whether VPS13 proteins might play a role in the formation of metabolic highly active tripartite contact regions between LDs, ER, and mitochondria.

In severe steatosis, PCP revealed that approximately 6% of the total proteome was targeted to LDs including kinases and transcription factors, thereby indicating new roles for LDs in influencing gene expression and signaling pathways. Interestingly, almost all proteins with lipid modifications such as Rab proteins or small G-proteins were detected on LDs. This sequestering of proteins to LDs might be induced by the dramatic expansion of LD surfaces. The increase in protein binding sites on LDs during steatosis might counteract the molecular crowding normally limiting LD binding to the proteins with highest affinities. Under these conditions, proteins with lower LD affinity that normally bind to other compartments might be retargeted to LDs. This led us to hypothesize that LD induced depletion of proteins from their initial localizations impairs cellular processes and the functions of other organelles and thereby contributes to the pathology and cellular dysfunction in hepatic steatosis.

A dramatic example for the sequestering of proteins to LDs was the surprising finding that all Golgi apparatus proteins cofractionating with LDs in the low-density fraction in the steatotic state. This was not caused by lipoprotein accumulation in the Golgi apparatus, as ApoB, the main very low density lipoprotein (VLDL) protein was predominantly localized to the ER. Confocal and super-resolution microscopy revealed that in the steatotic liver, the Golgi apparatus partially fragments and wraps around LDs, forming direct contacts with the LD monolayer (Figure 2A). This cellular reorganization was only observed under conditions of extreme LD accumulation in severe steatosis and not at earlier time points of high fat diet in the mouse liver. The Golgi-LD association was also not detected in most cell lines after lipid treatment, indicating that cellular models for steatosis do not completely reflect the more complex in vivo situation.

It still needs to be elucidated whether this reorganization of the secretory apparatus has a functional and protective role or whether it is simply a pathological consequence of extreme lipid accumulation impairing cellular functions. We speculate that close association between LDs and the Golgi apparatus could play a role in lipoprotein lipidation. So far, the assembly of the lipoprotein particles in hepatocytes is poorly understood and it is still enigmatic how lipids are mobilized and transferred from the cytosolic LDs to lipoprotein particles during their trafficking through the secretory apparatus. However, the observed general decrease in protein secretion in primary hepatocytes from steatotic mice points toward a pathological role. Indeed, parallel accumulation of lipids and impaired secretion could lead to a vicious cycle where more and more lipids accumulate. However, we note that the secretion defect

was relatively rapidly reversible by starvation. The key question is now whether the reorganization of the secretory apparatus induced by lipid accumulation is also happening in the liver of patients with nonalcoholic fatty liver disease and whether it plays a role for the pathology and the progression of the disease.

Potential Mechanism for the Golgi Apparatus Sequestering to LDs and Open Questions

What drives the relocalization of the Golgi apparatus proteins to LDs? All Golgi proteins float with LDs in the steatotic condition, no matter whether they have any structural features for LD binding or not. This favors the hypothesis that Golgi stacks or membrane fragments are sequestered to LDs via tethering proteins. Indeed, several crucial Golgi organizing proteins have structural features that would allow monolayer binding by either amphipathic helices or fatty acid modifications.

The golgins are interesting candidates to mediate these interactions (Figure 2B). This group of large coiled-coil proteins localizes to the Golgi apparatus via a C-terminal transmembrane domain or the interaction with small-GTPases (Munro, 2011). The golgins, expressed at high levels in the liver (according to our proteomic liver analysis), bind vesicles and direct them to the Golgi apparatus where they fuse with the bilayer. The vesicles are captured with mostly uncharacterized domains in their N-terminal part. One exception is GMAP210, which contains an N-terminal ALPS (amphipathic lipid packing sensor) motif that binds highly curved vesicles (Magdeleine et al., 2016). These ALPS domains sense lipid packing defects and are also able to bind to LDs whose monolayer has a greater extent of packing defects than bilayer membranes, especially under conditions of LD expansion when PLs are a limiting factor (Prevost et al., 2018). Both vesicles and LDs have many packing defects, a common surface feature that golgins use for selective binding and this might be the cause for a “mis-targeting” of golgins to LDs. This could be specifically enforced under conditions of major LD growth, when the expanded surface area provides new binding sites that compete proteins with potential LD binding sites off and sequester them. Golgins are important organizers for Golgi apparatus localizations and for instance, artificial targeting of GMAP210 leads to Golgi apparatus (GA) accumulation around mitochondria (Wong and Munro, 2014). A similar process could also occur on LDs in steatotic liver. Besides golgins, several other Golgi proteins contain ALPS domains (e.g., ARFGAP1, AKAP9, COG6) (Drin et al., 2007) or lipid modifications (Rab proteins, GRASP proteins) (Zhang and Wang, 2015) that in principle enable LD binding, making them further candidates to mediate the organelle attachment.

We observed that in steatotic liver, the protein profiles of subunits of the coat protein complex COPI for retrograde transport shifted dramatically and surprisingly overlapped with the peroxisomal profile. Previously, COPI has been found on peroxisomes by interacting with PEX11 α , a protein involved in peroxisome biogenesis at the ER when peroxisomal proliferation is induced by PPAR α agonists (Lay et al., 2006; Wiese et al., 2007). Indeed, under high fat diet, peroxisomal proliferation is induced and the question arises how COPI and Golgi apparatus relocalizations are connected or cause each other?

Another open question is the role of cellular signaling and posttranslational modifications for the lipid-induced organelle reorganization. The Golgi apparatus is a highly dynamic organelle whose assembly and reassembly during cell cycle is controlled by activation of kinases and phosphatases and phosphorylation status of Golgi organizing proteins (Valente and Colanzi, 2015). This process is initiated by mitotic kinases, including cyclindependent kinase 1 (CDK1), polo-like kinase-1 and 3 (PLK1,3). In addition, activation of RAF-MEK-ERK, JNK2 and CAMK-AMPK signaling have been found to control Golgi apparatus structure (Valente and Colanzi, 2015). The kinases phosphorylate Golgi stacking and tethering proteins (e.g., GORASP1 and 2, GM130) and thereby induce its fragmentation. In Alzheimer's disease, Golgi apparatus fragmentation is caused by increased GORASP1 phosphorylation by CDK5 (Gordon-Weeks, 2016). Therefore, investigating whether the activation of specific signaling pathways also plays a role in the loss of normal Golgi apparatus structure in hepatic steatosis is an important question, especially since strong changes in phosphorylation pattern of GA proteins occur in steatotic liver.

We believe that these and other interesting questions will soon become answerable with further technological progress in proteomics. We are especially excited about the prospect for drastically increased sensitivity leading to a reduction of input material required for proteomic and phosphoproteomic analysis. This will open the field to map organelle changes in response to metabolic challenges in various tissues and organs or even specific cell subtypes in in vivo disease models or human samples.

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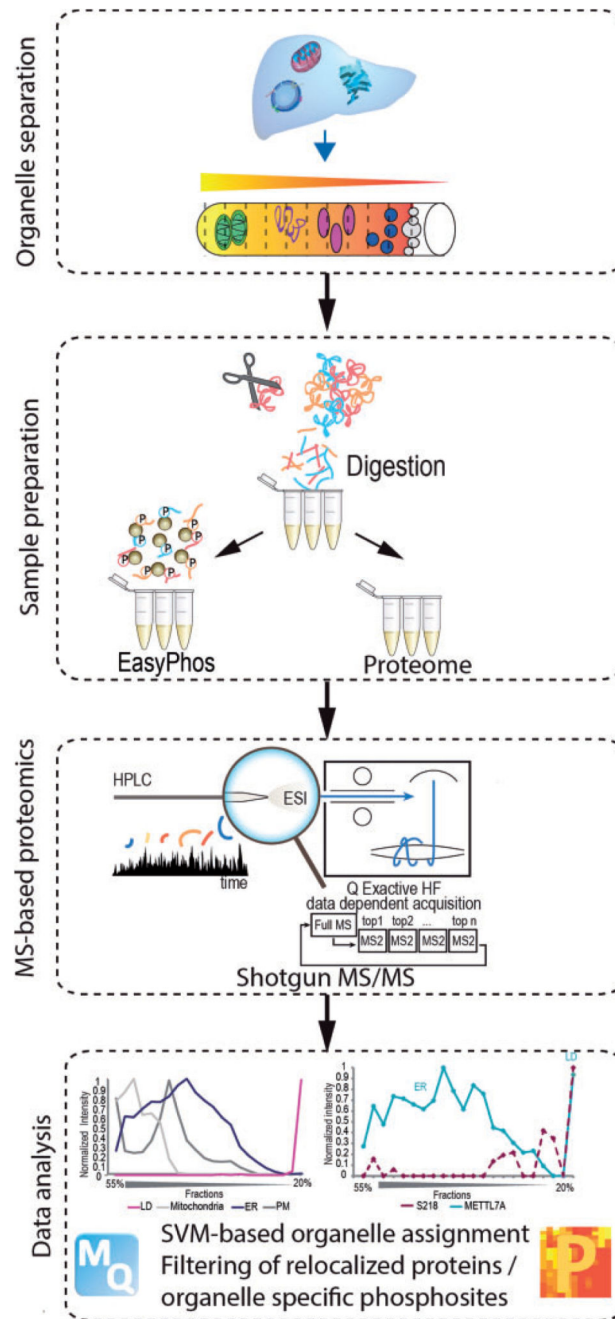


Figure 1.

Workflow for the characterization of organelle proteomes and LD contact sites by Protein Correlation Profiling (PCP). Organelles are separated by density-based or differential centrifugation and analyzed using proteomic and phosphoproteomic workflows. Protein and peptide profiles are generated by plotting abundances over organelle fractions. Subcellular localizations of proteins and peptides can be assigned using machine learning-based algorithms. LD proteins display a distinct and strongly separated peak in the lowest density fraction of the gradient. HPLC = high performance liquid chromatography; ER =

endoplasmic reticulum; LD = lipid droplet; ESI= electro spray ionization; SVM= support vector machines; HFX= hybrid quadrupole orbitrap mass spectrometry (MS).

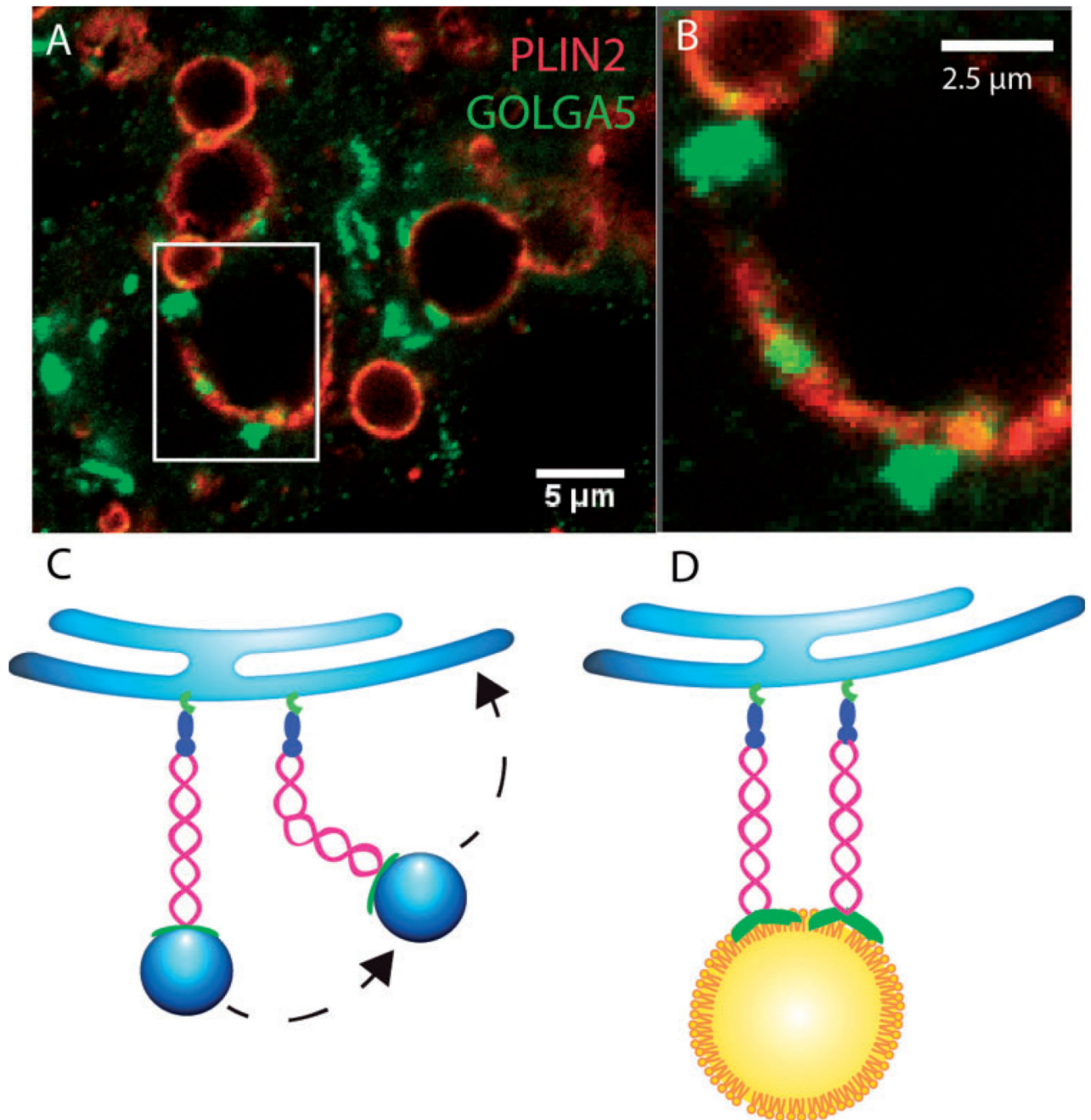


Figure 2. Potential mechanisms of the Golgi apparatus relocalization in steatotic liver (a) and (b) immunofluorescence microscopy of LDs stained via Perilipin2 (PLIN2) (red) and the Golgi apparatus via GOLGA5 (green) in the liver of steatotic mice showing the assembly of Golgi apparatus compartments around LDs. (c) and (d) Potential mechanism by which Golgi tethering proteins attach to LDs. (c) Under normal conditions Golgi tethering proteins bind to highly curved vesicles and direct them to the Golgi apparatus for fusion. (d) In steatotic

liver ttering proteins containing ALPS domains might mis-target to LDs induced by extreme LD accumulation and thereby increase organelle contacts.